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Varicella-Zoster Virus Immediate-Early 63 Protein Interacts with Human Antisilencing Function 1 Protein and Alters Its Ability To Bind Histones H3.1 and H3.3▽

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Varicella-zoster virus (VZV) immediate-early 63 protein (IE63) is abundantly expressed during both acute infection in vitro and latent infection in human ganglia. Using the yeast two-hybrid system, we found that VZV IE63 interacts with human antisilencing function 1 protein (ASF1). ASF1 is a nucleosome assembly factor which is a member of the H3/H4 family of histone chaperones. IE63 coimmunoprecipitated and colocalized with ASF1 in transfected cells expressing IE63 and in VZV-infected cells. IE63 also colocalized with ASF1 in both lytic and latently VZV-infected enteric neurons. ASF1 exists in two isoforms, ASF1a and ASF1b, in mammalian cells. IE63 preferentially bound to ASF1a, and the amino-terminal 30 amino acids of ASF1a were critical for its interaction with IE63. VZV IE63 amino acids 171 to 208 and putative phosphorylation sites of IE63, both of which are critical for virus replication and latency in rodents, were important for the interaction of IE63 with ASF1. Finally, we found that IE63 increased the binding of ASF1 to histone H3.1 and H3.3, which suggests that IE63 may help to regulate levels of histones in virus-infected cells. Since ASF1 mediates eviction and deposition of histones during transcription, the interaction of VZV IE63 with ASF1 may help to regulate transcription of viral or cellular genes during lytic and/or latent infection.

Varicella-zoster virus (VZV) is a neurotrophic human alphaherpesvirus. Primary infection causes chicken pox, or varicella, which results in a lifelong latent infection in trigeminal and dorsal root ganglia (30, 32, 35). Later in life, as a result of waning immune status due to aging or immunosuppression, VZV reactivates, resulting in shingles, or herpes zoster.

During latency, VZV expresses at least six different viral transcripts (11, 12, 25, 40). Open reading frame 63 (ORF63) is the most abundant VZV transcript expressed during latency (11). VZV ORF63 encodes a 278-amino-acid protein with immediate-early (IE) expression kinetics, referred to as IE63 (13). IE63 has been detected in latently infected human (18, 25, 36, 38) and experimentally infected rodent (13, 26) ganglia. While IE63 is predominantly expressed in the nucleus during lytic replication in vitro, during latency the protein is detected in the cytoplasm of sensory neurons (18, 36, 38).

IE63 is also abundantly expressed during lytic VZV replication (13, 28, 54). In VZV-infected cells, IE63 is extensively phosphorylated by VZV ORF47 protein kinase (27) and by cellular casein kinases (5, 54). IE63 is a component of the VZV tegument (28) and represses the activity of a number of VZV and heterologous viral and cellular promoters (5, 14). IE63 is required to overcome the host innate response mediated by alpha interferon (3) and to inhibit apoptosis in primary human neuronal cells infected with VZV in culture (24). IE63 binds to RNA polymerase II and VZV IE62, the major viral transactivator, and enhances the activity of the VZV gI promoter (37).

In this study, we show that VZV IE63 interacts with human antisilencing function 1 protein (ASF1) in transiently transfected or VZV-infected cells and that IE63 preferentially interacts with the ASF1a isoform. We locate the areas of ASF1 and IE63 important for this interaction and show that IE63 enhances the interaction of ASF1 with histones H3.1 and H3.3. To our knowledge, VZV IE63 is the first viral protein known to interact with human ASF1.

MATERIALS AND METHODS

Cells and viruses. MeWo (human melanoma) cells were provided by Charles Grose at the University of Iowa, Iowa City, IA, and were maintained in minimal essential medium (Invitrogen, Calsburg, CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), penicillin, streptomycin, and glutamine (Invitrogen). HeLa, U2OS, and COS cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). HeLa S 3.1 and HeLa S 3.3 cells, which express carboxyl-terminal FLAG and hemagglutinin (HA) epitope-tagged H3.1 and H3.3 (56), were kindly provided by Yoshihiro Nakatani at the Dana-Farber Cancer Institute (Boston, MA). U2OS, HeLa, HeLa S 3.1 and 3.3, and COS cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, penicillin, streptomycin, and glutamine. Recombinant Oka VZV (ROka) was obtained from cos-

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mid clones derived from the vaccine Oka strain of VZV (10). ROka-T7, ROka63D, ROka63-5 M, ROka63-10 M, ROka63-AccI, and ROka63-KpnI have been reported previously (8, 9, 33). All viruses were propagated and titrated in MeWo cells.

Plasmids. Plasmids expressing myc-tagged human ASF1a (p408) and ASF1b (p542) under the simian virus 40 and T7 promoters were described previously (61). Plasmids expressing myc-tagged chimeras of human ASF1a and ASF1b, including ASF1b-a-b-a (p601), ASF1a-b (p941), and ASF1b-a (p942), have been reported previously (57, 61). ASF1b-a-b (p1051) and ASF1 a-b-a (p1052) were constructed by using standard molecular biology methods, and details are available on request (P. D. Adams and M. Poustovoitov, unpublished data). Plasmid VZV IE62 (pCMV62 [10]) has been reported previously. Plasmids pcDNA63-5M and pcDNA63-10M (5) were obtained from Sébastien Bontems and Catherine Sadzot-Delvaux at the University of Liège, Liège, Belgium. pCI63, which expresses full-length VZV IE63, was constructed by PCR amplification using primer FP63NheI (5′-GCCTAGCTAGCTACCATGTTTTGCACCTCACCGGC-3′) and primer RP63NotI (5′-GTCAGAATGCGGCCGCATAAAGACTACACGC CATGGG-3′), digestion of the PCR product with NheI and NotI, and insertion of the DNA into the corresponding sites of pCI (Promega, Madison, WI). pCI-HA63, which expresses full-length IE63 with an amino-terminal HA tag, was constructed by PCR amplification using primer FP63HANheI (5′-GCCTAGCT AGCTACCATGTACCCATACGATGTTCCAGATTACGCTTTTTGCACCT CACCGGC-3′) and primer RP63NotI, digestion of the PCR product with NheI and NotI, and insertion of the DNA into the corresponding sites of pCI. pCI-HA63KpnI, which encodes the amino-terminal 208 amino acids of VZV IE63 with an amino-terminal HA tag, was constructed by PCR amplification using primer FP63HAEcoRI (5′-CGGAATTCACCATGTACCCATACGATGTTCC AGATTACGCTTTTTGCACCTCACCGGCT-3′) and primer RP63SalI (5′ GGAGTCGACTTAATTAATTATAGTACCCCGTCTGGTTCACAAGAATC GGT), digestion with EcoRI and SalI, and insertion of the DNA into the corresponding sites of pCI. All PCR amplifications were carried out using the Expand high-fidelity PCR system (Roche Applied Science, Indianapolis, IN), with VZV cosmid MstIIA (10) as the template. The sequences of all constructs were confirmed by restriction digestion and direct sequencing of the VZV DNA inserts. Plasmid pmaxGFP (Amaxa, Gaithersburg, MD) was used as a control for transient transfections.

Yeast two-hybrid analysis. Matchmaker GAL4 two-hybrid system 3 (BD Biosciences, Palo Alto, CA) was used to find binding partners for VZV IE63. Portions of VZV IE63 were cloned into pGBKT7, containing the GAL4 DNA binding domain, which served as the bait vector. A HeLa cell cDNA library fused to the GAL4 activation domain in plasmid pACT2 was used as the prey vector. VZV IE63 constructs containing nearly full-length IE63 (amino acids 3 to 278), the amino terminus of VZV IE63 (amino acids 1 to 165), and the carboxy terminus of VZV IE63 (the last 163 amino acids) were made. The nearly full-length construct (beginning at amino acid 3 of VZV IE63) was made by PCR of the VZV IE63 gene by using primers 63FstartEco (5′ GGACGAATTCTGCA CCTCACCGGCTACGCG 3′) and 63FstopPst (5′ ATTTCTGCAGACTACAC GCCATGGGGGGGC 3′), the amino-terminal construct by using primers 63Fstart Eco and 63N3PBam (5′ GACCCAGGATCCTAGCTATCGTCTTCA CCACCATC 3′), and the carboxy-terminal construct (which begins at amino acid 116) by using primers 63C5 PEco (5′ GCTTGAATTCACCCAGACGCA GTGCTTACGCG 3′) and 63FstopPst. The PCR products were digested with EcoRI and PstI (nearly full-length VZV IE63 and the carboxy terminus of VZV IE63) or EcoRI and BamHI (the amino terminus of VZV IE63) and inserted into the EcoRI-PstI or EcoRI-BamHI site of pGBKT7. The plasmids were then transfected into *Saccharomyces cerevisiae* strains AH109 and CG-1945. The sequences of all constructs were confirmed by restriction digestion and direct sequencing of the VZV DNA inserts.

Immunoprecipitations and immunoblots. Cells were lysed in either CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} buffer (1% CHAPS [T. J. Baker, Phillipsburg, NJ] in Tris-buffered saline [19.98 mM Tris, 136 mM NaCl, pH 7.4]) or radioimmunoprecipitation assay buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.1 mM dithiothreitol, 1% Nonidet P-40) supplemented with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 30 min on ice, and the clarified lysate was used for immunoprecipitations. Mouse monoclonal antibodies to VZV IE63 (9A12 [26]), ASF1a (MPH7 or MPB1, generated from mice immunized with GST-ASF1a by using standard procedures [23]), or anti-myc 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal antibody to ASF1a (antibody 87 [61]) was added to lysates (300 to 400 μl) and incubated on ice for 1 h, and antigen-antibody complexes were incubated with protein G-Sepharose beads overnight. The beads were washed several times with lysis buffer (without protease inhibitors) and boiled for 5 min in 1× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer. Proteins were separated on 4 to 20% Tris-glycine SDS-polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes, and incubated with rabbit polyclonal antibodies to VZV ORF61 protein (44), IE62 (44), IE63 (44), or ORF4 protein (42); mouse monoclonal antibodies to VZV glycoprotein E (Chemicon, Temecula, CA) or VZV IE63 (9A12); rabbit polyclonal antibody to human ASF1 (53); a cocktail of rabbit antibodies to Asf1a and Asf1b (antibody 87, which immunoprecipitates ASF1a [not ASF1b] but recognizes both human ASF1a and ASF1b in immunoblots, and antibody 88, which recognizes human ASF1a only [not ASF1b] in both immunoprecipitations and immunoblots [61]); or mouse monoclonal antibodies to myc (9E10), FLAG (M2; Sigma-Aldrich, St. Louis, MO), or HA (16B12; Covance, Emeryville, CA). After being washed, the membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody or horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL).

Transient-transfection assays. COS cells, grown in six-well plates, were transfected with 3.2 μg of plasmid DNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, the cells were harvested and lysed, and the clarified lysate was subjected to immunoprecipitation and/or immunoblotting. Transient transfection of HeLa S 3.1 and 3.3 cells was performed using Nucleofection (Amaxa) according to the manufacturer's recommendations. Briefly, 2×10^6 cells were suspended in 100 μl of solution L, 2 μg of DNA was added, and nucleofection was performed using the V-001 program. Immediately after nucleofection, 500 μl of medium containing 10% FBS was added and transferred to a single well of a 12-well plate containing 2 ml of medium with 10% FBS. Two days after nucleofection, the cells were harvested for immunoprecipitation.

Latent and lytic VZV infection of guinea pig enteric ganglia. Enteric ganglia were isolated from male guinea pigs as described previously (6). Briefly, the longitudinal muscle was dissected from the small intestine, together with the myenteric plexus, to which it adheres. The resulting preparation was digested with collagenase and triturated to dissociate smooth muscle and ganglia. Individual ganglia were isolated with a pipette, pooled, and cultured for 1 week in the presence of mitotic inhibitors. Cultures contained an average of 1,800 neurons per 4-cm^2 well. The mitotic inhibitors were then removed, and ganglia were inoculated with cell-free or cell-associated VZV at ~2,400 PFU/4-cm^2 well and cultured in Dulbecco's modified Eagle's medium-F12 medium supplemented with 2% FBS, amphotericin B, and gentamicin for 3 days. The cultures contained enteric neurons and glial cells but were nearly free of fibroblasts or smooth muscle cells. Cell-free VZV was prepared from infected human embryonic lung fibroblasts by sonication as described previously (22).

Confocal microscopy. HeLa cells grown on glass coverslips were transfected with plasmid DNA by using Lipofectamine 2000, and 24 h later, the cells were washed once with phosphate-buffered saline (PBS), fixed with 2% formaldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA) for 20 min, and permeabilized with methanol for 15 min at −20°C. The cells were washed three times with PBS, blocked with 20% goat serum in PBS for 1 h, and incubated with mouse anti-myc monoclonal antibody (immunoglobulin G_1 [IgG_1]; Santa Cruz) and rat anti-HA monoclonal antibody (3F10; Roche Applied Science), followed by Alexa Fluor 594 goat anti-mouse IgG_1 (Molecular Probes, Eugene, OR) and Alexa Fluor 488 goat anti-rat IgG (Molecular Probes). Guinea pig enteric ganglion neurons were fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 4), washed with PBS, blocked with goat serum, and permeabilized with 0.1% Triton X-100. The cells were stained with mouse monoclonal antibody to IE63 (9D12 [26]), rabbit anti-ASF1a antibody (Proteintech Group, Inc., Chicago, IL), and guinea pig neuron-specific ubiquitin carboxyl-terminal hydrolase L1 (PGP 9.5; Chemicon) overnight at 4°C. After being washed, the cells were incubated with goat anti-mouse IgG labeled with Alexa Fluor 594 (Molecular Probes), goat anti-rabbit IgG labeled with Alexa Fluor 488 (Molecular Probes), and goat anti-guinea pig IgG biotin (KPL, Gaithersburg, MD), with avidin-conjugated Alexa 680 and bisbenzimide (Sigma-Aldrich) used to label DNA. Coverslips were mounted using Prolong gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen) and examined by confocal microscopy.

RESULTS

Yeast two-hybrid screening shows that VZV IE63 interacts with human antisilencing function 1 protein. To better understand the function of VZV IE63, we used the yeast two-hybrid system to identify cellular proteins that interact with VZV IE63 protein. Of the three VZV IE63 constructs we generated, the nearly full-length VZV IE63 construct (amino acids 3 to

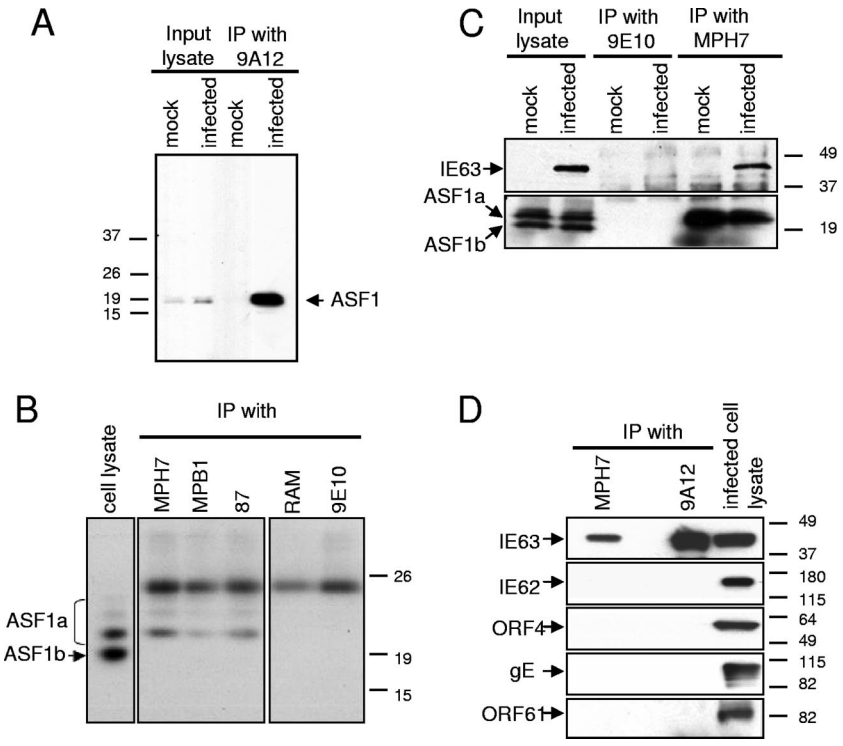

FIG. 1. Interaction of VZV IE63 with human antisilencing function 1 protein (ASF1) in VZV-infected cells. Human melanoma (MeWo) cells uninfected or infected with VZV ROka were lysed at 48 h postinfection, and equal amounts of lysates were incubated with mouse anti-VZV IE63 (9A12) (A and D), mouse anti-ASF1a (MPH7, IgG_1) (C and D), or mouse anti-myc 9E10 (IgG_1) (B and C) antibodies. Immune complexes were precipitated and immunoblotted with rabbit anti-ASF1 antibody which detects human ASF1a and ASF1b (A and C); rabbit anti-VZV IE63 antibody (C and D); rabbit antibody to IE62, ORF4 protein, or ORF61 protein; or mouse monoclonal antibody to gE (D). IP, immunoprecipitation. (B) Characterization of anti-ASF1a mouse monoclonal antibodies MPH7 and MPB1. U2OS cell lysates were immunoprecipitated with mouse monoclonal antibodies to ASF1a (MPH7 and MPB1), rabbit polyclonal antibody to ASF1a (antibody 87), or control antibodies (rabbit anti-mouse polyclonal antibody [RAM] or mouse anti-myc monoclonal antibody [9E10]). Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and then immunoblotted with a cocktail of purified rabbit polyclonal antibodies to ASF1a and ASF1b (antibodies 87 and 88). The left lane shows whole-cell extract of U2OS cells.

278) was not stable in yeast, while yeast containing the carboxy terminus of VZV IE63 grew very poorly; therefore, only the amino-terminal VZV IE63 construct was used for yeast two-hybrid screening. The bait vector containing amino acids 1 to 165 of VZV IE63 fused to the yeast GAL4 DNA binding domain and a prey vector containing HeLa cell cDNA fused to the yeast GAL4 activation domain were transfected into yeast strains AH109 and CG-1945. Yeast two-hybrid screening yielded a clone with a 1.3-kb insert in yeast strain AH109 and a second clone with a 1.7-kb insert in yeast strain CG-1945. Both were in-frame fusions containing the entire antisilencing function 1b (ASF1b) gene. Additional yeast two-hybrid experiments were performed using another construct, encoding amino acids 1 to 208 of ORF63, and ASF1a was found to interact with the new ORF63 construct.

VZV IE63 interacts with human ASF1 in VZV IE63-transfected and VZV-infected cells. To verify that VZV IE63 interacts with ASF1 in VZV-infected cells, human melanoma (MeWo) cells were either not infected or infected with VZV ROka for 48 h and lysed in 1% CHAPS buffer, and VZV IE63 was immunoprecipitated with monoclonal antibody 9A12. Immune complexes were analyzed by immunoblotting with rabbit anti-ASF1 serum, and ASF1 was found to coimmunoprecipitate with VZV IE63 (Fig. 1A). Mouse monoclonal antibody MPH7 was used in subsequent experiments; to verify that this antibody recognizes ASF1a, we immunoprecipitated cell lysates with monoclonal antibody MPH7 or MPB1 to ASF1a or rabbit antibody 87 to ASF1a. MPH7, MPB1, and 87 immunoprecipitated a polypeptide of ~23 kDa when blotted with rabbit antibodies 87 and 88, which corresponded to ASF1a but not ASF1b (Fig. 1B). Control immunoprecipitations with rabbit anti-mouse antibody or isotype control IgG_1 antibody (9E10) did not yield ASF1. These results demonstrate that monoclonal antibodies MPH7 and MPB1 and polyclonal antibody 87 immunoprecipitate ASF1a but not ASF1b. To verify the IE63-ASF1 interaction, equal amounts of either uninfected or VZV-infected MeWo cell lysates were incubated with MPH7 or isotype-matched control antibody, followed by immunoprecipitation and immunoblotting with rabbit anti-VZV IE63 polyclonal serum (Fig. 1C). VZV IE63 coimmunoprecipitated with anti-ASF1a antibody in VZV ROka-infected cells but not with the isotype-matched control antibody. To determine whether the interaction of ASF1 is specific for VZV IE63 and confirm that other viral proteins do not interact with ASF1, the MPH7 immune complexes from VZV ROka-infected lysates were immunoblotted with antibodies to VZV IE63, IE62, ORF4 protein, gE, or ORF61 protein. Only VZV IE63 coimmunoprecipitated with ASF1 (Fig. 1D). No interaction was detected

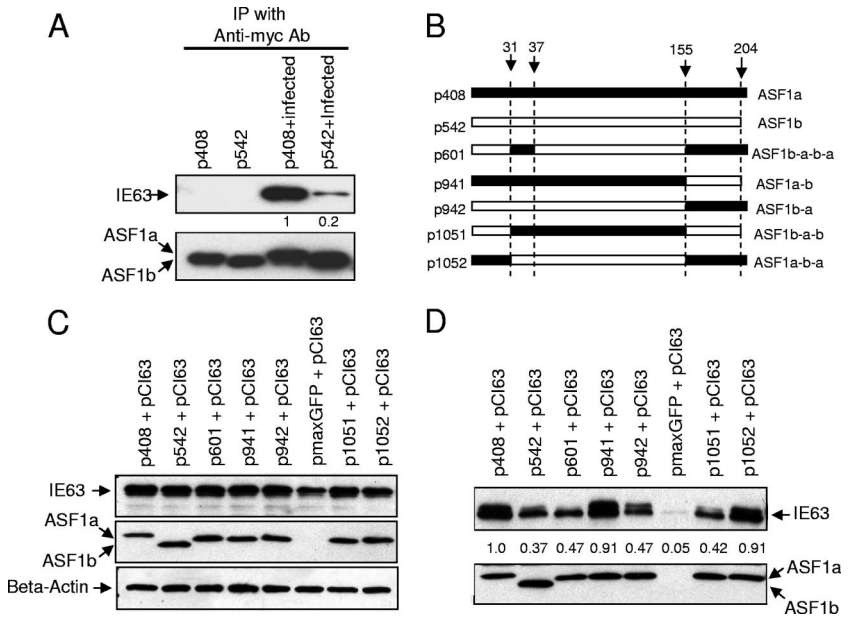

FIG. 2. Interaction of VZV IE63 with human ASF1a and ASF1b. (A) MeWo cells were transiently transfected with plasmids expressing myc-tagged human ASF1a (p408) or human ASF1b (p542). At 24 h posttransfection, the cells were infected with VZV ROka-T7, and 48 h postinfection, the cells were lysed and the clarified lysates were incubated with mouse anti-myc antibody (Ab). The immune complexes were precipitated and immunoblotted with rabbit anti-ASF1 or rabbit anti-VZV IE63 antibodies. IP, immunoprecipitation. (B) ASF1a, ASF1b, and chimeric ASF1 constructs used to identify the region of ASF1a important for binding to VZV IE63. (C) COS cells were transiently transfected with plasmids expressing myc-tagged, full-length ASF1a (p408), ASF1b (p542), and ASF1a and ASF1b (p601, p941, p942, p1051, or p1052) chimeras or pmaxGFP together with a plasmid expressing full-length VZV IE63 (pCI63). At 48 h posttransfection, the cells were lysed and equal amounts of clarified lysates were immunoblotted with mouse anti-myc monoclonal antibody (9E10), mouse anti-beta-actin antibody (clone AC-15; Sigma-Aldrich), or rabbit anti-VZV IE63 antibodies. (D) Equal amounts of lysates (comparable to those used in panel B) were incubated with mouse anti-myc antibody, and immune complexes were precipitated and immunoblotted with rabbit anti-VZV IE63 antibody or mouse anti-myc antibody. The numbers below the IE63 panel indicate the intensities of the IE63 bands relative to those in cells transfected with ASF1a (p408), normalized to the intensities of the corresponding ASF1 bands on the basis of densitometry by using ImageJ software (http://rsb.info.nih.gov/ij/). The IE63 doublets in Fig. 2D were seen when plasmids expressing IE63 were transfected into COS cells (also seen in Fig. 3C) but not into HeLa cells (Fig. 5C) and are likely due to differences in cell type used.

between IE63 and IE62, in contrast to prior studies (4, 40); this is likely due to differences in the lysis buffers and immunoprecipitation conditions used here.

VZV IE63 interacts preferentially with human ASF1a. Since the human genome carries two ASF1 genes, we determined if VZV IE63 shows preferential binding to ASF1a or ASF1b. MeWo cells were transfected with plasmid constructs expressing myc-tagged ASF1a (p408) or ASF1b (p542), and 24 h after transfection, the cells were infected with VZV ROka-T7. At 48 h after infection, the cells were lysed, and myc-tagged ASF1a and ASF1b were immunoprecipitated using anti-myc monoclonal antibody. Immunoblotting using anti-VZV IE63 antibodies demonstrated that VZV IE63 preferentially bound to ASF1a rather than to ASF1b (Fig. 2A). No cross-reactivity of anti-myc antibody with IE63 was detected (data not shown).

The amino-terminal 30 amino acids of ASF1 determine the preferential binding of VZV IE63 to ASF1a. Human ASF1a and ASF1b share 71% amino acid identity. The most conserved area of the two proteins, between amino acids 37 and 155, shows 90% identity, while amino acids 1 to 30 share 70% identity. Like VZV IE63, the histone chaperone HIRA preferentially binds to ASF1a compared to ASF1b (61). Since ASF1a amino acids 31 to 37 and 155 to 204 are important for the preferred interaction of HIRA with ASF1a compared to ASF1b (61), we tested whether similar amino acids of ASF1a are also important for its interaction with VZV IE63. COS cells were cotransfected with a plasmid expressing VZV IE63 (pCI63) and with a plasmid expressing myc-tagged ASF1a, ASF1b, or a chimera of the two proteins (Fig. 2B). Immunoblotting of cell lysates showed similar amounts of IE63 and ASF1 in each of the lysates (Fig. 2C). Immunoprecipitation of lysates with anti-myc monoclonal antibody and immunoblotting with rabbit anti-63 serum showed that ASF1a (p408), ASF1a-b (p941), and ASF1a-b-a (p1052) bound best to IE63 (Fig. 2D). These results indicate that amino acids 1 to 30 of ASF1 are especially important for binding to IE63. In contrast, amino acids 1 to 30 of ASF1b, which are present in constructs p542, p601, p942, and p1051, bound less strongly to IE63 than the other constructs.

VZV IE63 amino acids 171 to 208 and putative phosphorylation sites of VZV IE63 are important for the interaction of IE63 with ASF1a. The observation that the yeast two-hybrid system detected ASF1b by using a bait vector containing amino acids 1 to 165 of VZV IE63, while ASF1a was detected with a bait vector containing amino acids 1 to 208 of VZV IE63, suggests that amino acids in the region of amino acids 166 to 208 of IE63 may be important for binding to ASF1a. To define the region of VZV IE63 that binds to ASF1a, we used a panel of VZV ROka IE63 mutants. MeWo cells were infected with VZV ROka (which expresses full-length IE63), ROka63-KpnI

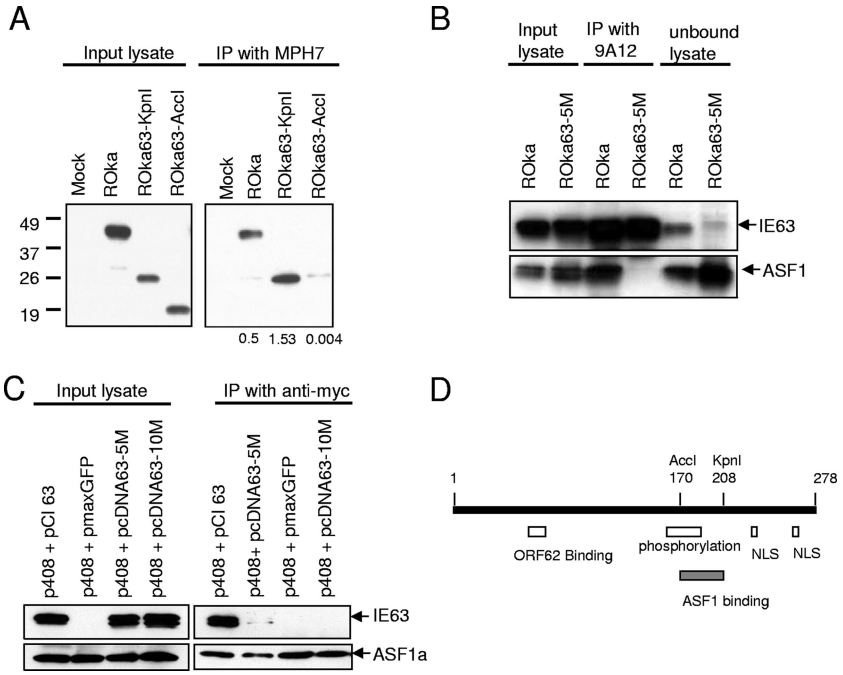

FIG. 3. Amino acids 170 to 280 and putative phosphorylation sites of VZV IE63 are important for the interaction with ASF1. (A) Equal numbers of MeWo cells that were uninfected or infected with VZV ROka, ROka63-KpnI, or ROka63-AccI were lysed at 48 h postinfection, and equal amounts were immunoblotted with rabbit anti-VZV IE63 antibody. Another aliquot of each lysate was incubated with mouse anti-ASF1a monoclonal antibody MPH7, and the immunoprecipitates were immunoblotted with rabbit anti-VZV IE63 antibody. The faint bands in the ROka (lane 6) and ROka63-AccI (lane 8) cells at 26 kDa are due to the light chain of the MPH7 antibody. IP, immunoprecipitation. (B) ASF1 interacts very weakly with IE63-5M in ROka63-5M-infected MeWo cells. MeWo cells infected with VZV ROka or ROka63-5M were lysed at 48 h postinfection. An aliquot of each lysate was incubated with mouse anti-VZV IE63 (9A12) monoclonal antibody, and immune complexes were recovered on protein G-Sepharose beads and eluted. A portion of cell lysate (input), eluted immune complexes, and a portion of supernatant remaining after immunoprecipitation (unbound lysate) were subjected to immunoblotting using rabbit anti-VZV IE63 antibody and rabbit anti-ASF1 antibody. (C) COS cells were transiently transfected with a plasmid expressing myc-tagged, full-length ASF1a (p408) and plasmid pCI63, pmaxGFP, pcDNA63-5M, or pcDNA63-10M. At 48 h posttransfection, the cells were lysed and equal amounts of clarified lysates were immunoblotted with mouse anti-myc antibody (to detect ASF1a) and rabbit anti-VZV 63 antibody. Another aliquot of each lysate was incubated with mouse anti-myc antibody, and the immune complexes were immunoblotted with rabbit anti-VZV IE63 or mouse anti-myc antibodies. (D) Map showing ASF1 binding site and other domains of VZV IE63. "NLS" are nuclear localization signals, and "phosphorylation" is the region which contains phosphorylation sites mutated in ROka63-5M.

(which expresses amino acids 1 to 208), or ROka63-AccI (which expresses amino acids 1 to 170), and equal amounts of cell lysates were analyzed by immunoblotting for IE63 expression (Fig. 3A, left panel). Immunoprecipitation of equal amounts of the same cell lysates with antibody to ASF1a followed by immunoblotting for IE63 showed that IE63 from cells infected with VZV ROka or ROka63-KpnI, but not ROka63-AccI, bound to ASF1a (Fig. 3A, right panel). This result explains the different outcome of the yeast two-hybrid data and indicates that residues 171 to 208 of IE63 are required for its interaction with ASF1a.

VZV IE63 is a highly phosphorylated protein, and its phosphorylation status affects VZV replication in vitro and the ability of VZV to establish latency in rodents (9). To determine if putative phosphorylation sites of IE63 affect its interaction with ASF1a, MeWo cells were infected with VZV ROka or VZV ROka63-5M (which has point mutations in five putative phosphorylation sites in IE63), and equal volumes of cell lysates were analyzed by immunoprecipitation with monoclonal antibody to IE63, followed by immunoblotting for ASF1 (Fig. 3B). IE63 from cells infected with ROka coimmunoprecipitated ASF1, but little or no ASF1 was bound to IE63-5M (Fig. 3B, lanes 3 and 4). This result was further supported by the large amount of ASF1 remaining in the supernatant after immunoprecipitation (unbound lysate) from the ROka63-5M-infected cell lysate compared to the level for the ROka-infected cell lysate (Fig. 3B, lanes 5 and 6).

ROka63-10M (which has point mutations in 10 putative phosphorylation sites in IE63) is even more impaired for replication than ROka-5M, and we were unable to infect cells and obtain lysates with similar levels of VZV protein expression. Therefore, to determine the ability of IE63-10M to interact with ASF1, we cotransfected COS cells with a plasmid expressing myc-tagged ASF1a and a plasmid expressing IE63 with point mutations in 10 putative phosphorylation sites in IE63 (pDNA63-10M [5]), a plasmid expressing IE63 with point mutations in five putative phosphorylation sites in IE63 (pcDNA63-5M), or a plasmid expressing IE63 with wild-type IE63 (pC163). Immunoblotting of cell lysates showed that all of the IE63 proteins were expressed at similar levels; however, immunoprecipitation of the lysates with antibody to myc (which precipitates myc-tagged ASF1) showed that ASF1a had a weaker interaction with both IE63 mutants than with wild-type IE63 (Fig. 3C). Taken together, these data indicate that

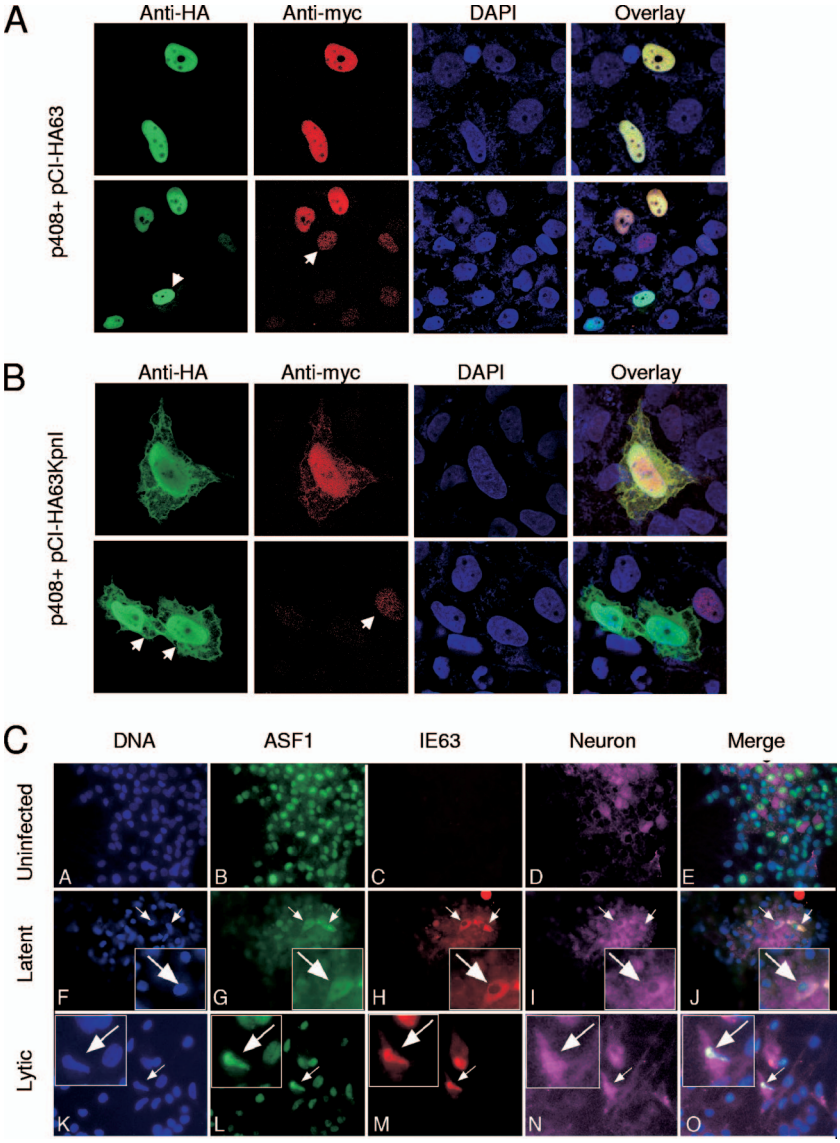

FIG. 4. Subcellular localization of ASF1 and VZV IE63. (A and B) HeLa cells were cotransfected with plasmid p408 expressing myc-tagged ASF1a and either plasmid pCI-HA63 expressing HA-tagged, full-length VZV IE63 (A) or pCI-HA63KpnI, which expresses the HA-tagged amino-terminal 208 amino acids of VZV IE63 (B). At 48 h posttransfection, the cells were fixed, permeabilized, and stained with mouse anti-myc monoclonal antibody and rat anti-HA monoclonal antibody, followed by Alexa Fluor 488 (green)-conjugated goat anti-rat IgG antibody, Alexa Fluor 594 (red)-conjugated goat anti-mouse IgG_1 antibody, and DAPI. The arrows in panels A and B indicate cells expressing only ASF1a or IE63. (C) Cultured guinea pig enteric neurons were uninfected (top row) or infected with either cell-free (middle row) or cell-associated (bottom row) VZV, and at 3 days postinfection, the neurons were fixed, permeabilized, and stained with mouse anti-IE63 monoclonal antibody 9D12, rabbit anti-ASF1a antibody, and guinea pig anti-neuron specific ubiquitin carboxyl-terminal hydrolase L1 antibody, followed by goat anti-rabbit Alexa Fluor 488 (green), goat anti-mouse Alexa Fluor 594 (red), and biotin labeled anti-guinea pig antibody, with avidin-conjugated Alexa 680 (purple) and bisbenzimide used to stain DNA (blue).

amino acids 171 to 208 of IE63 and putative phosphorylation sites of IE63 are both important for its interaction with ASF1a (Fig. 3D).

VZV IE63 colocalizes with ASF1 in mammalian cells. IE63 is located predominantly in the nucleus during lytic replication of VZV, while the protein is present exclusively in the cytoplasm of sensory neurons during latency. When the virus reactivates from latency, IE63 is detected in both the nucleus and the cytoplasm. These observations suggest that subcellular localization of IE63 may play an important role in VZV latency. ASF1 localizes predominantly to the nuclei of cells (41). Therefore, we hypothesized that IE63 and ASF1 might colocalize in the nucleus during VZV lytic replication, while during latency, when IE63 is in the cytoplasm, at least a portion of ASF1 might localize with IE63 in the cytoplasm. HeLa cells were cotransfected with plasmid pCI-HA63, which expresses IE63 under the cytomegalovirus IE promoter, and plasmid p408, which expresses a low level of myc-tagged ASF1a under the simian virus 40 promoter. ASF1a colocalized with IE63 in the nucleus (Fig. 4A, upper row). Most transfected cells ex-

pressed both IE63 and ASF1a; however, a few cells expressed only ASF1a or IE63 (Fig. 4A, lower row). ASF1a also colocalized with IE63 in HeLa cells transfected with plasmid p408 and infected with VZV ROka (data not shown).

We next determined whether alteration of IE63 localization in the cell changes the localization of ASF1a. IE63 contains two nuclear localization signals near its carboxyl terminus. Transfection of a plasmid with a deletion in both of the nuclear localization domains of IE63 into Vero or HeLa cells was shown to result in localization of the truncated protein predominantly in the cytoplasm (4, 5). Transfection of plasmid pCI-HA63KpnI, which expresses the HA-tagged amino-terminal 208 amino acids of IE63 (and lacks both nuclear localization signals but still binds to ASF1a) into HeLa (Fig. 4B) and Vero (data not shown) cells showed that the protein localized predominantly in the nucleus but showed more cytoplasmic staining than wild-type IE63. Cotransfection of plasmids pCI-HA63KpnI and p408 into HeLa cells showed that ASF1 localized to both the nucleus and the cytoplasm in a manner similar to that of HA63KpnI. Therefore, ASF1a colocalizes with IE63 whether the latter is solely in the nucleus or partially in the cytoplasm.

When guinea pig enteric neurons are infected with cell-associated virus in vitro, VZV induces a lytic infection, during which IE63 is localized in the nucleus. In contrast, when enteric neurons are infected with cell-free virus, VZV establishes a latent infection, during which IE63 is confined to the cytoplasm (6, 17). We compared the cellular localizations of ASF1 and IE63 in guinea pig enteric neurons latently or lytically infected with VZV. Cells were stained with antibodies to ASF1, IE63, a neuronal cell marker (ubiquitin carboxyl-terminal hydrolase L1), and with bisbenzimide used to label DNA. ASF1 localized to the nucleus in uninfected enteric neurons (Fig. 4C, panels A to E). In contrast, during latent infection of enteric neurons with cell-free VZV, IE63 localized to the cytoplasm, while ASF1 showed nuclear and increased cytoplasmic staining (Fig. 4C, panels F to J). In contrast, during lytic infection of enteric neurons with cell-associated VZV, IE63 was predominantly localized to the nucleus and colocalized with ASF1 (Fig. 4C, panels K to O). Colocalization of IE63 and ASF1a in one or more subcellular compartments is consistent with a functional link between the two proteins.

VZV IE63 enhances the interaction of ASF1 with histone H3.1 and H3.3. ASF1 interacts with histones H3 and H4 and plays a major role in nucleosome assembly and disassembly during DNA replication, transcription, and DNA repair (43). Human ASF1a and ASF1b interact with histone H3 isotypes H3.1 and H3.3 (56). To determine if binding of IE63 to ASF1 interferes with the interaction of ASF1 with H3, we transfected HeLa S cells stably expressing HA-tagged H3.1 or H3.3 with a plasmid expressing VZV IE63 or green fluorescent protein. Immunoprecipitation of cell lysates with anti-HA antibody, followed by immunoblotting with rabbit anti-ASF1 antibody, showed increased amounts of ASF1a and ASF1b interacting with H3.1 or H3.3 in cells expressing IE63 compared to the level for cells expressing green fluorescent protein (Fig. 5A). No IE63 was detected when the same immunoblots were probed with anti-IE63 antibodies, indicating that H3.1/H3.3-ASF1 complexes do not contain detectable levels of IE63 (data not shown). To confirm that the effect of IE63 on the interaction of H3.1 and ASF-1 was specific for IE63, we transfected HeLa S 3.1 cells with empty vector, pCI63, or pCMV62 (which expresses VZV IE62) and found that only IE63 enhanced the interaction of ASF1 with H3.1 (Fig. 5B).

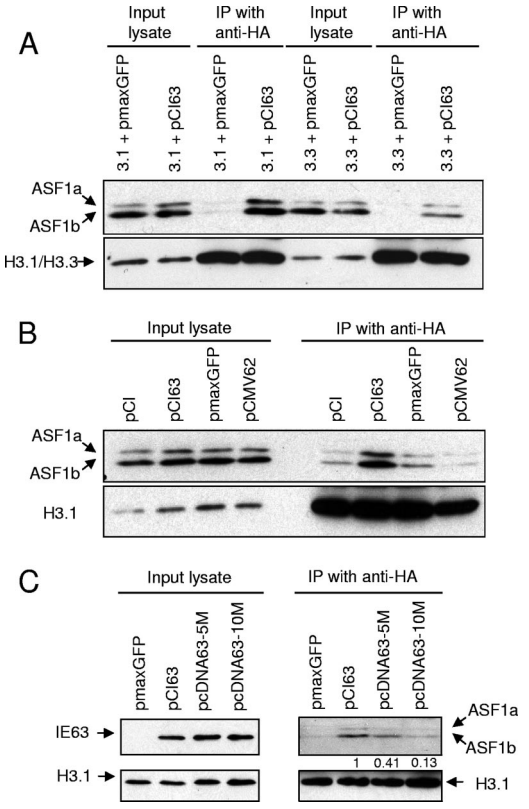

FIG. 5. Effect of VZV IE63 on the interaction of ASF1 with histones H3.1 and H3.3. (A) HeLa S 3.1 and HeLa S 3.3 cells, which express C-terminal HA-tagged H3.1 and H3.3, respectively, were nucleofected with plasmid pmaxGFP or pCI63, lysed, and immunoblotted with rabbit anti-ASF1 antibody or mouse anti-HA antibody (to detect H3.1 and H3.3). Another aliquot of each lysate was incubated with mouse anti-HA antibody, and the immune complexes were precipitated and immunoblotted with rabbit anti-ASF1 antibody or mouse anti-HA antibody. IP, immunoprecipitation. (B) HeLa S 3.1 cells were nucleofected with plasmid pCI, pCI63, pmaxGFP, or pCMV62; lysed; and immunoblotted with rabbit anti-ASF1 or mouse anti-HA antibody. Another aliquot of each lysate was incubated with mouse anti-HA antibody, and the immune complexes were precipitated and immunoblotted with rabbit anti-ASF1 antibody or mouse anti-HA antibody. (C) HeLa S 3.1 cells were nucleofected with plasmid pmaxGFP, pCI63, pcDNA63-5M, or pcDNA63-10M; lysed; and immunoblotted with rabbit anti-VZV IE63 or mouse anti-HA antibody. Another aliquot of each lysate was incubated with mouse anti-HA antibody, and the immune complexes were precipitated and immunoblotted with rabbit anti-ASF1 antibody or mouse anti-HA antibody. The intensities of the ASF1 bands were quantified using ImageJ software (http://rsb.info.nih.gov/ij/) and normalized to the intensities of the corresponding IE63 bands in the input lysate, and this ratio is shown below the ASF1 bands.

Since ASF1a showed a weaker interaction with IE63 putative phosphorylation site mutants than with wild-type IE63, we tested if these mutants fail to enhance the interaction of ASF1 and H3.1. HA-tagged H3.1 was coimmunoprecipitated with ASF1a and ASF1b, and the levels of the ASF1 proteins were normalized to the amount of IE63 expressed in cells transfected with pCI63,

pCDNA63-5M, or pcDNA-10M. There was 2.5-fold less ASF1a and ASF1b coimmunoprecipitated with H3.1 in cells expressing IE63-5M and 7.7-fold less ASF1a and ASF1b coimmunoprecipitated with H3.1 in cells expressing IE63-10M than in cells expressing wild-type IE63 (Fig. 5C).

DISCUSSION

We have identified and characterized the interaction of VZV IE63 with human ASF1, an evolutionary conserved nucleosome assembly factor which belongs to the H3/H4 family of histone chaperones. ASF1 was initially reported as a protein whose overexpression in budding yeast *Saccharomyces cerevisiae* derepresses gene expression (31). ASF1, together with chromatin assembly factor (CAF1) and histones H3 and H4, assembles nucleosomes onto replicating DNA (58). In addition to CAF1, ASF1 interacts with a wide range of chromatin-associated proteins, such as histone cell cycle regulation defective homolog A (HIRA) and TATA box binding protein-associated factor TAFII-250 (7, 21). ASF1 also associates with DNA damage checkpoint kinases, such as Tousled-like checkpoint kinase and Rad53 kinase (15, 53). As expected for a protein that interacts with multiple other proteins, ASF1 has been implicated in various cellular functions, including DNA replication, gene transcription, and cellular response to DNA damage, all of which involve orderly eviction and deposition of histones onto DNA (reviewed in reference 43).

We found that VZV IE63 specifically interacts with ASF1 in both transiently transfected and VZV-infected cells. Two isoforms of ASF1, ASF1a and ASF1b, are found in mammals. We have shown that IE63 interacts preferentially with ASF1a rather than ASF1b. In contrast to mammal cells, *Drosophila*, chicken, yeast, and *Xenopus* cells have one copy of ASF1. Deletion of ASF1 in *Drosophila* and chicken cells results in accumulation of the cells in S phase, with decreased DNA replication and apoptosis (49, 50). Similarly, knockdown of ASF1 expression in human cells by small interfering RNA (siRNA) also results in accumulation of the cells in S phase (19). Deletion of ASF1 in yeast is not lethal but results in an altered global chromatin structure (2), decreased acetylation of lysine 56 of histone H3 (48), increased levels of endogenous DNA damage (45, 46), decreased stability of stalled replication forks (16), defective transcription-coupled histone eviction and deposition (1, 55), and defects in heterochromatin-mediated silencing of telomeres and mating loci (29, 51). Human ASF1a and ASF1b genes encode proteins of 204 amino acids (22.9 kDa) and 202 amino acids (22.4 kDa), respectively. Human ASF1a and ASF1b show 71% identity at the amino acid level. They are highly conserved over much of their length but diverge at their amino and carboxyl termini. Both human ASF1a and human ASF1b bind the p60 subunit of CAF1 (57). In contrast, HIRA, like VZV IE63, preferentially binds to ASF1a (57). HIRA, the human ortholog of the yeast proteins that also contribute to transcriptionally silent heterochromatin in yeast (52), competes with CAF1 for binding to ASF1a (57). ASF1a and HIRA, together with H3 and H4, mediate DNA synthesis-independent nucleosome assembly, both in vitro (47, 56) and in vivo (34, 60).

We found that the amino-terminal 30 amino acids of ASF1a determine the preferential binding of VZV IE63 to ASF1a rather than ASF1b. Using chimeric ASF1a-ASF1b proteins, Tang and coworkers showed that the amino-terminal 30 amino acids and carboxyl-terminal 50 amino acids of ASF1a and ASF1b determine the different affinities of these proteins for binding to HIRA (57). According to the model for the association of ASF1 with HIRA and histone H3/H4, both amino and carboxyl termini of ASF1a contribute to its binding to HIRA. At least part of the HIRA-binding site is located on the opposite side of the H3 binding site of ASF1 (57). We found that binding of IE63 to ASF1 did not alter the latter's interaction with HIRA (A. P. Ambagala and J. I. Cohen, unpublished data).

We have shown that VZV IE63 lacking the carboxyl-terminal 70 amino acids binds to ASF1a similarly to wild-type IE63. In contrast, IE63 lacking the carboxyl-terminal 108 amino acids or IE63 which contains mutations in several putative phosphorylation sites is impaired for binding to ASF1a. Since a VZV mutant in which IE63 lacks the carboxyl-terminal 70 amino acids is not impaired for replication in cell culture or for latency in rodents, while IE63 lacking the carboxyl-terminal 108 amino acids or IE63 with mutations in several putative phosphorylation sites is impaired for replication and for latency (9), the interaction of IE63 with ASF1 may play a critical role in VZV replication and establishment of latency. We studied VZV replication in human diploid fibroblasts in which expression of both ASF1a and ASF1b were knocked down by gene-specific siRNA. Despite knockdown of over 90% of both ASF1a and ASF1b on immunoblot analysis, VZV replication, measured by virus titers and plaque sizes, remained unchanged (Ambagala and Cohen, unpublished). This might be due to insufficient knockdown of ASF1 expression in human diploid fibroblasts. This possibility is supported by the observation by Groth and colleagues (19; A. Groth, personal communication) in which suboptimal knockdown of ASF1a and ASF1b was inadequate for a clear effect of ASF1 on cell cycle progression to be observed. Alternatively, the failure of ASF1 knockdown to inhibit VZV replication could be due to another histone chaperone compensating for reduced ASF1 levels in the cells, or the interaction of ASF1 with IE63 may not be critical during lytic VZV replication in vitro.

We found that IE63 colocalizes with ASF1 in both VZV-infected and IE63-transfected cells. Both endogenous and ectopically expressed ASF1 proteins are distributed homogenously throughout the nucleus (41, 59). ASF1 exists in a soluble nuclear fraction, and unlike HIRA and CAF1, ASF1 is not tightly associated with chromatin (41). IE63, like ASF1, also is distributed diffusely in the nuclei of transiently transfected or VZV-infected cells in vitro. Cells transfected with IE63 lacking the carboxyl-terminal 70 amino acids, which include two putative nuclear localization signals (4, 5), show a modest increase in the amount of IE63 in the cytoplasm. Interestingly, ASF1 expression was also increased in the cytoplasm of cells transfected with the carboxy-terminal IE63 mutant. Furthermore, using the guinea pig enteric neuron latency model of VZV, we demonstrated that during latency, IE63 sequesters a portion of ASF1 into the cytoplasm. The interaction of IE63 with ASF1 in this setting might contribute to the ability to establish or maintain latent VZV infection.

We observed an increase in binding of H3 variants H3.1 and H3.3 to ASF1 when IE63 was present. Histones are highly

basic proteins that form complexes with DNA. Excess free histones interfere with chromosome segregation and inhibit cell growth (20, 39). Therefore the interaction of ASF1 with H3/H4 may bind up excess newly synthesized H3/H4 and thereby act as a buffer to prevent potentially toxic effects from an excess of free histones (19, 20).

In summary, we have identified regions of ASF1 and IE63 important for their interaction. VZV lacking IE63 (ROka63D) or having mutations in IE63 that impair its interaction with ASF1 (e.g., ROka63-AccI and ROka63-5M) is impaired for latency in rodents (8, 9). Therefore, the interaction of IE63 and ASF1 may play a major role in cellular and/or viral gene expression during latent infection.

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